

TMH/KMM:jam 02/03/06 480082 EP03-008C-US
PATENT

Attorney Reference Number 6616-72626-01
Application Number 10/633,279

AMENDMENTS TO THE SPECIFICATION

Please replace paragraph [008] with the following rewritten paragraph:

Globulins and albumins are common seed storage proteins in dicotyledonous plants (dicots). These proteins can be distinguished from each other based on differential solubility; albumins are water soluble, whereas globulins are soluble in salt solutions. In many dicots, including citrus and almond (*Prunus amygdalus*), 12S globulins are the most prevalent seed storage proteins. 12S globulins are multimeric proteins composed of dimer subunits. Each dimer contains a 30-40 kDa alpha-subunit and a 20 kDa beta-subunit. The dimer subunits are derived from a single precursor polypeptide that undergoes several post-translational modifications, including ~~eleaveage~~cleavage of a signal peptide followed by cleavage of the pre-protein into the alpha and beta subunits.

Please replace paragraph [010] with the following rewritten paragraph:

The invention is directed to a PRU promoter having seed-associated promoter activity that comprises in 3' to 5' direction, the following regions: (a) a core promoter sequence containing a TATA-box, and (b) a sequence ~~confering~~conferring seed specificity that contains a motif with similarity to the legumin-box consensus CATGCATG. In some embodiments the PRU promoter additionally comprises (c) an upstream sequence motif which is predicted to function as an enhancer element. In some embodiments, the PRU promoter is bi-directional, having a complementary strand that also comprises regions (a) and (b), and optionally (c). In one aspect of the invention, the bi-directional PRU promoter is used in a double-stranded plant expression vector that comprises, in the 5' to 3' orientation, a first heterologous protein encoding sequence in the antisense direction, the PRU promoter, and a second heterologous encoding sequence in the sense direction, wherein the PRU promoter directs seed-associated expression of both the first and the second heterologous protein encoding sequences.

Please replace paragraph [011] with the following rewritten paragraph:

Figures 1A and 1B show an alignment of the complete nucleotide sequence of the ChPRU promoter (top strand in Fig. 1; SEQ ID NO:1) with its reverse complement (bottom strand; SEQ ID NO:6). The following features are indicated in bold, italicized text for each

TMH/KMM:jam 02/03/06 480082 EP03-008C-US
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sequence: (1) the putative TATA-box, (2) motif with similarity to the legumin-box consensus CATGCATG, and (3) an upstream sequence motif common to both orientations of the chPRU promoter. Each of these features is associated with regions of high identity between the ChPRU promoter sequence and its reverse complement, indicated by underlining, and are predicted to function as (1) core promoter sequences, (2) sequences ~~confering~~conferring seed-specificity, and (3) enhancer elements.

Please replace paragraph [014] with the following rewritten paragraph:

The isolated promoter was found to direct high-level seed-associated gene expression when operably lined to heterologous coding sequences. As used herein, the terms "isolated PRU promoter" and "chPRU promoter" refer to a nucleic acid comprising the sequence provided in SEQ ID NO:1 or the reverse complement thereof, SEQ ID NO:6. The term also encompasses fragments and derivatives ~~thereof~~thereof that retain seed-associated promoter activity as discussed in more detail below. As used herein, the term "seed-associated promoter" refers to a promoter that directs RNA synthesis at higher levels in seeds than in other cells and tissues. A "seed-specific" promoter is a seed-associated promoter that directs RNA synthesis essentially only in the seed. But, under certain conditions and using particular detection methods, very low levels of expression in tissue other than seed may be detected from a seed-specific promoter.

Please replace paragraph [019] with the following rewritten paragraph:

It is expected that fragments of the disclosed chPRU promoter sequences that retain seed-associated promoter activity will minimally comprise a core promoter domain and a domain that confers seed-specificity. These regions are contained within nucleotides 1055-1212 of SEQ ID NO:1, and nucleotides 1043-1198 of SEQ ID NO:6. Thus, in one embodiment of the invention, the derivative chPRU promoter is uni-directional and comprises a nucleotide sequence selected from nucleotides 1055-1212 of SEQ ID NO:1, and nucleotides 1043-1198 of SEQ ID NO:6. Preferably, the derivative chPRU promoter additionally comprises a ~~putative~~putative enhancer domain, and thus comprises a nucleotide sequence selected from nucleotides 854-1212 of SEQ ID NO:1, and nucleotides 827-1198 of SEQ ID NO:6.

TMH/KMM:jam 02/03/06 480082 EP03-008C-US
PATENT

Attorney Reference Number 6616-72626-01
Application Number 10/633,279

Please replace paragraph [020] with the following rewritten paragraph:

Figure 1 can be referenced for guidance in making derivative chPRU sequence that share less than 100% identity with SEQ ID NOs: 1 and 6, and fragments thereof. In general, regions that share high identity with an alignment of SEQ ID NO:1 (top strand) and its reverse complement (bottom strand; SEQ ID NO:6) are expected to contribute to the seed-associated promoter function and should generally be conserved in derivative chPRU promoter sequences. These regions are underlined in Figure 1. Regions of lower identity are expected to be unnecessary for the seed-associated promoter function, and thus can vary substantially in corresponding derivative sequences, e.g. they can vary in length (i.e. can serve as spacer regions of varying length) and/or can vary in sequence identity. The sequences within SEQ ID NO:1 that are predicted to function as (1) core promoter sequence, (2) ~~sequence conferring~~ seed-specificity, and (3) enhancer element, as shown in Figure 1, are set forth in SEQ ID NOs: 7-9, respectively. And, the sequences within SEQ ID NO:6 that are predicted to function as (1) core promoter sequence, (2) ~~sequence conferring~~ seed-specificity, and (3) enhancer element, are set forth in SEQ ID NOs: 10-12, respectively.

Please replace paragraph [026] with the following rewritten paragraph:

The invention is directed to chimeric genes (expression cassettes) comprising nucleotide sequences of the isolated PRU promoter, or a functionally active derivative thereof, operably linked to and controlling the expression of a heterologous gene. The terms "chimeric gene" and "expression cassette" refer to a protein encoding nucleotide sequence comprising sequences that are heterologous with respect to each other (i.e., not naturally occurring together). A chimeric gene of the present invention comprises a PRU promoter sequence that is heterologous with respect to the protein encoding sequences. Thus, a chimeric gene may comprise a protein encoding sequence that is native to a plant, but heterologous with respect to the operably-linked promoter. Thus, the heterologous gene can be any gene other than chPRU1 or chPRU2. As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, which may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons). The term "gene"

TMH/KMM:jam 02/03/06 480082 EP03-008C-US
PATENT

Attorney Reference Number 6616-72626-01
Application Number 10/633,279

may be used interchangeably with the terms "heterologous nucleic acid coding sequence" or the term "protein encoding sequence."

Please replace paragraph [031] with the following rewritten paragraph:

In addition to a chimeric gene or gene inhibition construct comprising a PRU promoter, vectors of the present invention may comprise other functional sequences. Exemplary sequences include selectable marker genes that permit the selection of transformed plant cells by rendering the cells resistant to an amount of an agent that would be toxic to non-transformed plant cells. Exemplary selectable marker genes include the neomycin phosphotransferase (*nptII*) resistance gene, hygromycin phosphotransferase (*hpt*), bromoxynil-specific nitrilase (*bxn*), phosphinothricin acetyltransferase enzyme (BAR) and the spectinomycin resistance gene (*spt*), wherein the selective agent is kanamycin, hygromycin, geneticin, the herbicide glufosinate-ammonium ("Basta") or spectinomycin, respectively. Vectors may contain further sequences that allow for selection and propagation in a secondary host, such as an origin of replication and a selectable marker sequence. Typical secondary hosts include bacteria and yeast. In one embodiment, the secondary host is *Escherichia coli*, the origin of replication is a *colE1*-type, and the selectable marker is a gene encoding ampicillin resistance. Vectors of the invention may further comprise sequences that facilitate the integration of chimeric genes into plant chromosomes, such as regions of the Ti plasmid of *Agrobacterium tumefaciens*. Binary Ti-based vector systems that may be used to transfer polynucleotides are known to those of skill in the art, and many are commercially available (e.g., pBI121 Clontech Laboratories, Palo Alto, CA).

Please replace paragraph [045] with the following rewritten paragraph:

Various seed-associated traits are of interest. In one embodiment of the invention, a PRU promoter is used to ~~modify~~ modify the quantity and/or composition of carbohydrates, lipids (especially fatty acids), and/or amino acids in seed. Accordingly, heterologous genes of interest include those involved in oil, starch, carbohydrate and nutrient metabolism, as well as those affecting kernel size, sucrose loading, and the like. In one specific application of the invention, the brazil nut 2S albumin gene (Muntz et al., Nahrung 1998 Aug;42(3-4):125-7) is expressed in seed to alter amino acid composition. In other applications, lipid metabolism genes are

TMH/KMM:jam 02/03/06 480082 EP03-008C-US
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Application Number 10/633,279

expressed to alter the content and/or composition of seed oils. Exemplary lipid metabolism genes include desaturases (see, e.g., U.S. Pat. Nos. 5,552,306 and 5,614,393), acyl carrier proteins (ACPs), thioesterases, acetyl transacylases, acetyl-coA carboxylases, ketoacyl-synthases, malonyl transacylases, and elongases. Such lipid metabolism genes have been isolated and characterized from a number of different bacteria and plant species. Specific genes whose altered expression in seed has been shown to cause modified lipid phenotypes include diacylglycerol acyltransferase (Jako et al., 2001, Plant Physiol 2001, 126:861-74), modified ACP desaturase (Cahoon and Shanklin, 2000, Proc Natl Acad Sci U S A 97:12350-5), phytoene synthase (Shewmaker et al., 1999, Plant J 20:401-412), acetyl-coenzyme A carboxylase (Roesler et al., 1997, Plant Physiol 113:75-81), beta-Ketoacyl-CoA synthase (Lassner et al., 1996, Plant Cell 1996, 8:281-92), sn-2 acyltransferase (Zou et al., 1997, Plant Cell 9:909-23), acyl-ACP thioesterases and glycerolipid hydroxylase.

Please replace paragraph [061] with the following rewritten paragraph:

Fatty acid methyl ester (FAME) analysis. Fatty acid methyl esters were prepared from leaves and seeds of transformant lines carrying the three above-described constructs and controls lines (both wild type Col-0 and *fad2* mutant). Quantitative determination of leaf and seed fatty acid composition was performed as follows. Either whole seeds or cut leaves were transesterified in 500 μ l 2.5% H₂SO₄ in MeOH for 3 hours at 80 degrees C, following the method of Browse et al. (Biochem J 235:25-31, 1986) with modifications. A known amount of heptadecanoic acid was included in the reaction as an internal standard. 750 μ l of water and 400 μ l of hexane were added to each vial, which was then shaken vigorously and allowed to phase separate. Reaction vials were loaded directly onto GC for analysis, and the upper hexane phase was sampled by the autosampler. Gas chromatography with Flame Ionization detection was used to separate and quantify the fatty Acid methyl esters. Agilent 6890 Plus GC's were used for separation with Agilent Innowax columns (30m x 0.25mm ID, 250um film thickness). The carrier gas was Hydrogen at a constant flow of 2.5 ml/ minute. 1ul of sample was injected in splitless mode (inlet temperature 220 °C, Purge flow 15ml/min at 1 minute). The oven was programmed for an initial temperature of 105°C, Initial Time 0.5 minutes, followed by a ramp of 60°C per minute to 175°C, a 40°C /minute ramp to 260°C with a final hold time of 2 minutes. Detection was by Flame Ionization (Temperature 275°C, Fuel flow 30.0 ml/min, Oxidizer 400.0

TMH/KMM:jam 02/03/06 480082 EP03-008C-US
PATENT

Attorney Reference Number 6616-72626-01
Application Number 10/633,279

ml/min). Instrument control and data collection and analysis was using the Millennium Chromatography Management System (Version 3.2, Waters Corporation, Milford, MA). Integration and quantification was performed automatically by the Millennium software.